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INFLUENCE OF NON-STEROID ANTI-INFLAMMATORY AND IMMUNOSUPPRESSIVE DRUGS ON HEPATIC TRYPTOPHAN PYRROLASE ACTIVITY IN RATS*

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Abstract—High oral doses of the anti-inflammatory drugs indomethacin, acetylsalicyclic acid, sodium salicylate, salicylamide, mefenamic acid, flufenamic acid, amidopyrine, phenylbutazone and benzydamine administered repeatedly, did not influence tryptophan pyrrolase activity in livers of intact rats. The nonresponsiveness of tryptophan pyrrolase was in contrast to a stimulation of tyrosine aminotransferase caused by flufenamic acid.

Induction of tryptophan pyrrolase due to hydrocortisone was not inhibited generally by non-steroid drugs; exceptions were flufenamic acid and benzydamine which depressed hormonal induction. The authors' recent statement is confirmed that inhibition of protein synthesis due to induction is no essential property of non-steroid anti-inflammatory drugs.

The immunosuppressive drugs cyclophosphamide, triaziquone and azathioprine increased tryptophan pyrrolase activity, probably due to an inhibition of enzyme degradation; chloroquine, 6-mercaptopurin, 6-azauridin and amethopterin did not influence enzyme activity. Induction of tryptophan pyrrolase due to hydrocortisone was depressed by azathioprine, 6-mercaptopurin, 6-azauridin and amethopterin.

ENZYME inhibition by non-steroid anti-inflammatory drugs has been observed by many workers.[1] Speculation on the possible significance of enzyme inhibition plays an important role in discussions on the mechanism of action of anti-inflammatory drugs.[2] Much less is known about increases of enzyme activities due to anti-inflammatory drugs and their eventual relevance with respect to antiphlogistic effects.

In a recent communication, we reported the failure of the majority of a series of clinically used antirheumatic drugs to induce microsomal drug-metabolizing enzymes in rat liver.[3] Among antirheumatic drugs, enzyme induction of the phenobarbital type seems to be limited to pyrazolone derivatives.[3,4]

The present paper deals with the glucocorticoid-type of enzyme induction. Till now, the anti-inflammatory and antirheumatic efficacy of corticosteroids has not been reached, at least clinically, by any non-steroid anti-inflammatory drug. The mechanism of the anti-inflammatory action of steroids has not been clarified, nor has it for non-steroids. During the past 10 years, however, biochemistry has elucidated the mechanism of numerous metabolic glucocorticoid effects, referring them to enzyme induction.[5] Although the relevance of enzyme induction with respect to the anti-inflammatory

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mechanism of steroids has yet to be established, it seemed reasonable to search for corresponding biochemical actions in the group of non-steroid anti-inflammatory drugs.

Tryptophan pyrrolase (EC 1.13.1.12) belongs to the best-investigated glucocorticoid-inducible enzymes.[6] Apparently, there are relations between tryptophan metabolism and inflammation which are detailed elsewhere.[7] 5-Hydroxytryptamine is a proved inflammation mediator.[8] Glucocorticoids decrease the tissue content of 5-hydroxytryptamine, probably due to an increased flow rate of tryptophan along the oxidative pathway initiated by tryptophan pyrrolase.[9] Skidmore and Whitehouse[10] demonstrated by studies *in vitro* that non-steroid anti-inflammatory drugs decrease 5-hydroxytryptamine formation by an inhibition of L-tryptophan hydroxylase. It was not known whether non-steroids stimulate oxidative tryptophan metabolism, too. Houck *et al.*[11] found that the induction of collagenolytic enzymes in the skin of rats due to certain non-steroid anti-inflammatory drugs followed the same kinetics as tryptophan pyrrolase induction in rat liver due to hydrocortisone.

It was mainly for the cited facts that we decided to choose tryptophan pyrrolase for our study. The present paper aimed to find out whether non-steroid anti-inflammatory drugs increase hepatic tryptophan pyrrolase activity *in vivo* and/or influence enzyme induction due to hydrocortisone. Because of the growing importance of immunosuppressive drugs in the treatment of rheumatic diseases, some alkylating and antimetabolic agents were included into the investigations.

EXPERIMENTAL

Animals and treatment schedule. The experiments were performed with colony-bred male Wistar rats (Jena) aged 30–35 days, in one experiment aged 80 days. The animals were fed a pelleted standard diet (manufactured by "Arbeitsgruppe Versuchstierzucht Berlin") and tap water *ad lib.* Room temperature was 22–24°, relative air humidity $>50\%$. 4–5 days prior to experimental use, the animals were randomized. As a rule, six groups of animals were formed for each experiment, two of them receiving non-steroid drugs, one receiving hydrocortisone, two receiving the non-steroid drugs together with hydrocortisone, and a control group receiving drug vehicle. In a limited number of experiments, only one drug was tested, in these cases only 4 animal groups were used. All compounds were administered p.o. via stomach tube in 1 ml 1% tylose solution per 100 g body wt. Drugs were given 28, 16 and 4 hr before sacrificing. This regimen was used according to Houck *et al.*[11], who administered drugs 28 and 4 hr before decapitation. The administration at 16 hr before killing was inserted in order to obtain more permanent blood levels. The single doses of the nonsteroid drugs were, with few exceptions, 20 per cent of the acute p.o. LD_{50}. Sources of the drugs, single doses and references of the dosages used are listed in Table 1.

Hydrocortisone* was administered 28, 16 and 4 hr before decapitation in all experiments. The single dose was 5 mg/kg (free alcohol), in one experiment 10 mg/kg. If hydrocortisone and drugs were given simultaneously, both were suspended in the same sample of vehicle.

* Hydrocortisone was obtained from VEB Jenapharm Jena.

TABLE 1

Drugs	Drug sources or purity, respectively	Single doses (mg/kg)	Reference No. of the LD_{50} or of the dosage used
Indomethacin	Sharp & Dohme, Münche§	3	12
Sodium salicylate	DAB 7, GDR	120*	13
Acetylsalicylic acid	DAB 7, GDR	300	13
Salicylamide	DAB 7, GDR	160	
Amidopyrine	DAB 7, GDR	340	13
Phenylbutazone (sodium salt)	DAB 7, GDR	50*	13
Mefenamic acid	Parke, Davis & Co., Münche§	340	14
Flufenamic acid	Parke, Davis & Co., Münche§	90	15
Benzydamine × HCl	Tropon, Köl§	200	16
Chloroquine diphosphate	DAB 7, GDR	20	17
Cyclophosphamide	VEB Ankerwerk Rudolstadt	140	17
Amethopterin (methotrexat®)	Lederle, München	2	17
6-Mercaptopurine (mercaleukin®)	VEB Arzneimittelwerk Dresden	75	17
6-Azauridine (riboazauracil®)	Spofa, Praha	100	17
Triaziquone (trenimon®)	Bayer, Leverkusen	0·45†	
Azathioprine (imuran®)	Burroughs, Wellcome & Co., London	200†	

* 20% of the i.p. LD_{50}.

† This dosage was not derived from other authors; dates on acute toxicity in the rat did not come to our knowledge.

§ We would like to thank the manufacturers for kindly supplying this product.

Enzyme assay. In all experiments, animals were killed by neck fraction at 2.00 p.m. After exsanguination, the livers were removed quickly and samples of 1 g of liver tissue placed into ice-cold 0·15 M KCl. Homogenization and tryptophan pyrrolase assay in the homogenate were performed according to the method of Chiancone,[18] which was slightly modified as follows: the volume of the incubation mixture was doubled yielding a total of 10 ml, consisting of 1 ml 0·2 M phosphate buffer pH 7·0, 4 ml 0·14 M KCl in 0·0025 N NaOH, 1 ml 0·03 M L-tryptophan and 4 ml of a 10% liver homogenate (w/v). The enzyme activity was measured without adding hematin or ascorbic acid.[18] The incubation period was prolonged to 90 min. 30 and 90 min after starting the reaction, half of the incubation mixture was deproteinized, and kynurenin was determined spectrophotometrically from the difference between the 90 and 30 min absorbance values. Results are expressed as μmole kynurenine $\times$ hr^{-1} $\times$ 100 mg $protein^{-1}$. Protein was determined with the biuret method using crystalline bovine serum albumine as a standard. Hepatic tyrosine aminotransferase activity (EC 2.6.1.5) was determined according to Wurtman and Axelrod.[19]

RESULTS

Non-steroid anti-inflammatory drugs. Tryptophan pyrrolase activity in rat liver homogenate was not influenced by pretreatment *in vivo* with non-steroid anti-inflammatory drugs (Table 2). The poor rise caused by indomethacin was probably accidental.

TABLE 2. TRYPTOPHAN PYRROLASE ACTIVITY IN RAT LIVER HOMOGENATE AFTER TREATMENT *in vivo* WITH NON-STEROID ANTI-INFLAMMATORY DRUGS AND/OR HYDROCORTISONE

		Pretreatment			
		Control (1% tylose)	Hydrocortisone (3 × 5 mg/kg)	Drug alone (3 × 20% of the LD_{50})	Hydrocortisone (5 mg/kg) and drug (20% of the LD_{50}) combined, ×3
1‡	Indomethacin	100 ± 8.6 (1·98)	330 ± 20·3*	127 ± 7·9*	
2‡	Sodium salicylate	100 ± 3·7 (1·98)	217 ± 19·6*	84 ± 11·7	
3‡	Phenylbutazone	100 ± 10·2 (1·67)	226 ± 17·5*	101 ± 11·7	
4	Acetylsalicylic acid	100 ± 14·9 (2·32)	270 ± 20·1*	98 ± 14·7	214 ± 25·0
	Amidopyrine	100 ± 14·9 (2·32)	270 ± 20·1*	119 ± 12·9	211 ± 32·4
5	Mefenamic acid	100 ± 25·1 (1·44)	329 ± 37·2*	149 ± 25·1 [6]	434 ± 31·7
	Flufenamic acid	100 ± 25·1 (1·44)	329 ± 37·2*	100 ± 12·8	149 ± 34·1†
6	Salicylamide	100 ± 18·2 (1·41) [6]	271 ± 30·8*	90 ± 10·7	224 ± 32·2 [6]
	Benzydamine	100 ± 18·2 (1·41) [6]	271 ± 30·8*	103 ± 29·0	145 ± 17·4†
7§	Indomethacin	100 ± 10·4 (1·93)	359 ± 21·5*		284 ± 20·4
	Sodium salicylate	100 ± 10·4 (1·93)	359 ± 21·5*		285 ± 27·8
	Phenylbutazone	100 ± 10·4 (1·93)	359 ± 21·5*		363 ± 16·6
8	Flufenamic acid	100 ± 11·5 (2·21)	270 ± 20·6*	89 ± 10·6	141 ± 8·3†

Because of differences of the control values the results were normalized to per cent of control activities; control values are given additionally, as μmole kynurenine × hr^{-1} × 100 mg $protein^{-1}$ (values in parentheses). Means ± S.E.M.

* Significantly different from controls.

† Significantly different from hydrocortisone rats. The *t*-test was used for statistical comparison ($P \leq 0{\cdot}05$). The number of animals was 7 unless given separately in angular brackets. Experiments were listed according to the chronological sequence. In Exp. No. 8 flufenamic acid was reinvestigated in animals of another age class in order to exclude an age-dependence of the flufenamic acid effect observed in Exp. No. 5.

‡ In experiments No. 1–3 only 3 groups of rats were studied; the missing results were completed in Exp. No. 7.

§ These experiments supplement Exp. No. 1–3.

In all experiments, hydrocortisone caused a 2- to 3·5-fold increase of hepatic tryptophan pyrrolase activity. This induction was, in general, not influenced by non-steroid anti-inflammatory drugs. There were two exceptions, flufenamic acid and benzydamine almost abolishing the glucocorticoid effect. The inhibitory action of flufenamic acid could be reproduced in 80 day-old rats (see exp. No. 8 in Table 2), indicating that this effect was independent of the age of the animals. In a further experiment not listed in Table 2, flufenamic acid was administered p.o., whereas hydrocortisone was given i.p. Hydrocortisone was somewhat less effective after i.p. than p.o. administration. Inhibition of the inductory glucocorticoid effect of flufenamic acid was not significant in this experiment.

For reasons of comparison, hepatic tyrosine aminotransferase, another glucocorticoid-inducible enzyme, was assayed in rats treated orally with flufenamic acid and/or hydrocortisone. As shown in Table 3, tyrosine aminotransferase activity was clearly increased by flufenamic acid; the hydrocortisone effect was not depressed. This behaviour is in noticeable contrast to tryptophan pyrrolase.

TABLE 3

Control (1% tylose)	Hydrocortisone (3 × 10 mg/kg p.o.)	Flufenamic acid (3 × 90 mg/kg p.o.)	Hydrocortisone plus flufenamic acid p.o.
3·10 ± 0·22 (5)	6·35 ± 0·48* (6)	10·48 ± 2·08 (6)*	12·67 ± 1·84 (6)

Tyrosine aminotransferase activity (μmole *p*-hydroxyphenylpyruvate × 20 min^{-1} × 10 mg protein^{-1}) in rat liver after treatment *in vivo* with flufenamic acid and/or hydrocortisone. Symbols as in Table 2. In this experiment, hydrocortisone was used as the acetate ester. Liver samples were stored for 48 hr at −20° before tyrosine aminotransferase assay. Storage caused a slight decrease in enzyme activity, as compared with fresh liver.

Immunosuppressants. The immunosuppressive drugs cyclophosphamide, triaziquone and azathioprine increased tryptophan pyrrolase activity significantly. Chloroquine, amethopterin, 6-mercaptopurine and 6-azauridine did not change enzyme activity significantly at the dosage used (Table 4). Induction due to hydrocortisone was depressed by amethopterin, 6-mercaptopurine, 6-azauridine and, weakly, azathioprine. Cyclophosphamide, triaziquone and chloroquine did not significantly influence the hydrocortisone effect.

DISCUSSION

Non-steroid anti-inflammatory drugs did not induce tryptophan pyrrolase. This enzyme which is the rate limiting step in oxidative tryptophan metabolism[18] is involved in the regulation of relative turnover rates of tryptophan along other metabolic pathways, e.g. 5-hydroxytryptamine formation. Hormone-induced rise of tryptophan pyrrolase results in a decrease of the 5-hydroxytryptamine level in tissue, probably because of an enhanced flow of tryptophan via oxidative pathway.[9] This mechanism might play a role in the anti-inflammatory action of glucocorticoid hormones though, admittedly, the dosage administered in order to obtain enzyme induction in laboratory animals[9] considerably exceeds therapeutic corticosteroid doses

TABLE 4

Exp. No.	Drugs studied	Pretreatment: Control (1% tylose)	Pretreatment: Hydrocortisone (3 × 5 mg/kg)	Pretreatment: Drugs alone (3 × the dosage specified in Table 1)	Pretreatment: Hydrocortisone (5 mg/kg) and drugs together (3 times)
10	Chloroquine	100 ± 13·1 (1·13)	422 ± 27·3*	106 ± 14·8	430 ± 15·3
	Cyclophosphamide	100 ± 13·1 (1·13)	422 ± 27·3*	227 ± 22·4*	373 ± 50·9 [6]
11	Amethopterin	100 ± 7·9 (3·48)	361 ± 20·7*	117 ± 6·6	221 ± 17·3† [6]
	6-Mercaptopurine	100 ± 7·9 (3·48)	361 ± 20·7	138 ± 19·7	239 ± 11·6† [5]
12	Triaziquone	100 ± 15·4 (1·65)	333 ± 40·0*	186 ± 31·3* [6]	296 ± 23·2
13	6-Azauridine	100 ± 6·6 (1·14) [6]	517 ± 16·8*	130 ± 15·5	377 ± 29·4† [6]
	Azathioprine	100 ± 6·6 (1·14) [6]	517 ± 16·8	361 ± 45·4* [6]	412 ± 31·3† [6]

Tryptophan pyrrolase activity in rat liver homogenate after treatment *in vivo* with immunosuppressive drugs and/or hydrocortisone. For explanation see Table 2.

alleviating effectively complaints in rheumatic diseases in man.[20] However, enzyme-inducing doses correspond fairly well to doses effectively inhibiting adjuvant-induced arthritis in rats.[21] Thus, a correlation between anti-inflammatory and enzyme-inducing activity might exist, at least in the rat. As the above results suggest, the outlined mechanism does not play a role in the action of non-steroid anti-inflammatory drugs.

It should be emphasized that the failure of non-steroid anti-inflammatory drugs to affect tryptophan pyrrolase does not exclude other enzymes being induced by the same drugs in a hydrocortisone-like fashion, e.g. histidine decarboxylase of the rat stomach,[22] collagenolytic enzymes in rat skin,[11] and also hepatic tyrosine aminotransferase (cf. Table 3). Inhibition of the hydrocortisone-induced rise of tryptophan pyrrolase activity by flufenamic acid and benzydamine was initially referred to a deteriorated intestinal absorption of hydrocortisone. This view was favoured by several observations: both in fenamate- and benzydamine-treated animals stomachs were inflated maximally and, in some cases, stuffed with food. Moreover, fenamates produced considerable gastrointestinal hemorrhage. Finally, when hydrocortisone was administered i.p. instead of p.o., induction of tryptophan pyrrolase was not inhibited significantly. However, hydrocortisone dosage was higher when given intraperitoneally (hydrocortisone acetate, 10 mg/kg) so that the inhibitory effect of flufenamic acid was perhaps overplayed in this experiment. Results listed in Table 3 speak against a reduction of hydrocortisone absorption by flufenamic acid since tyrosine aminotransferase induction by hydrocortisone given orally was not inhibited by flufenamic acid given orally as well.

In general, the non-steroid anti-inflammatory drugs investigated did not influence tryptophan pyrrolase induction by hydrocortisone. This accords with our previous finding that induction of drug-metabolizing microsomal enzymes in rat liver by phenobarbital is not influenced by non-steroid anti-inflammatory drugs.[3] However, once again it must be pointed out that there are differences between inducible enzymes as to their responsiveness to non-steroid anti-inflammatory drugs. Thus, Grant and de Szöcs[23] found a parallelism between anti-inflammatory activity and inhibition of the induction of β-galactosidase in Klebsiella aerogenes, whereas Westphal *et al.*[24] did not find such an influence on the same enzyme in *Escherichia coli* K 12. Obviously, inhibition of enzyme induction which might be expected as a result of the uncoupling action[1] of the majority of non-steroid anti-inflammatory drugs is no general property of these compounds, and results obtained with one enzyme may be generalized only very cautiously as can be seen from the difference in response to treatment with flufenamic acid between tryptophan pyrrolase and tyrosine aminotransferase in the present investigations.

The immunosuppressants amethopterin, 6-mercaptopurine, 6-azauridine and azathioprine inhibited tryptophan pyrrolase induction by hydrocortisone, probably due to an inhibition of nucleic acid synthesis and secondarily protein synthesis. This mechanism of action is also assumed for 5-fluorouracil which inhibits both postnatal development and substrate induction of tryptophan pyrrolase.[25]

Cyclophosphamide, triaziquone and azathioprine increased tryptophan pyrrolase activity. This is an interesting action of substances inhibiting nucleic acid and protein synthesis. There are at least three possible mechanisms for explanation of this increase:[26] (1) enzyme activation; (2) enzyme *de novo* synthesis; and (3) inhibition of enzyme degradation. The failure of the drugs to increase the hydrocortisone effect is

hardly consistent with the assumption of an enzyme activating action. Also an increased de novo synthesis is unlikely. Induction of tryptophan pyrrolase by hydrocortisone is RNA-dependent and results from enzyme de novo synthesis.[27] As shown in Table 4, immunosuppressive drugs inhibited induction by hydrocortisone. This action on one hand and de novo synthesis due to the same drugs should exclude each other. Inhibition of enzyme degradation is the possible cause for the "induction" of tryptophan pyrrolase by histones.[28] A related mechanism, i.e. inhibition of a degrading enzyme analogous to the action of histones and actinomycin D[28] might account also for the rise of tryptophan pyrrolase activity elicited by cyclophosphamide, triaziquone and azathioprine. L-Tryptophan, inhibiting enzyme degradation too, potentiates the enzyme inducing effect of hydrocortisone.[25] The failure of immunosuppressive drugs to potentiate the action of hydrocortisone in our experiments may be caused by an inhibition of both enzyme degradation and synthesis resulting in only small net changes of the hydrocortisone effect.

One might be surprised at the enzyme stimulating effect of azathioprine and the absence of this effect from 6-mercaptopurine, the former compound being transformed to the latter *in vivo*. However, 6-mercaptopurine dosage was distinctly smaller on a molar base than azathioprine dosage, which might provide an explanation for the observed difference.

Considering the poor effects of non-steroid anti-inflammatory drugs on tryptophan pyrrolase, the behaviour of tyrosine aminotransferase was remarkable. The striking difference in response to drug treatment between the two glucocorticoid-inducible enzymes led to extended investigations on the influence of anti-inflammatory drugs on hepatic tyrosine aminotransferase, the results of which will be published in a following paper.

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Addendum—After finishing the manuscript a paper by A. A.-B. Badaway and M. J. H. Smith appeared (*Biochem. Pharmac.* **21,** 97 1972). The authors found an elevation of hepatic tryptophan pyrrolase activity after treatment of rats with salicylate. This increase was observed up to several hours after drug administration and was attributed to an increase in liver tryptophan due to a depletion of serum protein-bound tryptophan by salicylate. The failure to find a corresponding increase in our experiments probably depends upon the longer time interval between the first drug administration and enzyme assay (28 hr) in our investigations. At this time, protein-bound tryptophan is expectedly depleted completely, thus preventing another increase in hepatic tryptophan level following the last drug administration.